Investigation of Age-Related Changes in Expression of Aquaporin-1 and Aquaporin-3 in Rat Bone

Yaşlanmayla Sıçan Kemiğindeki Aquaporin-1 ve Aquaporin-3 Ekspresyonu Değişikliklerinin Araştırılması

The Effects of Aging on the Bone Aquaporins

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Özet

Amaç: Aquaporinler su ve iyon transportu sağlayan membran kanal proteinleridir. Yaşlanma ile dokulardaki aquaporinlerin miktarı değişmektedir. Aquaporin-1 (AQP1) ve aquaporin-3 (AQP3)'ün kıkırdak dokuda yaşlanma ile azaldığı gösterilmiştir. Ancak kemik dokuda yaşlanma ile aquaporinlerin miktarında değişim gösterilmemiştir. Bu çalışmada genç ve yaşlı hayvanların kemik dokularında AQP1, AQP3 ve tip I kollajen ekspresyonlarının immünohistokimyasal olarak araştırılması amaçlandı. Gereç ve Yöntem: Bu çalışmada 14 adet Wistar Albino cinsi sıçan kullanıldı. Grup I (n=7) iki aylık genç sıçanlardan, Grup II (n=7) onsekiz aylık yaşlı sıçanlardan oluşmaktaydı. Kemik dokusu (femur) histopatolojik ve immunohistokimyasal değerlendirme için alındı. Rutin histolojik prosedürün ardından parafine gömülen dokulardan 4-5 μm kalınlığında kesitler alındı. İmmunohistokimyasal olarak AQP1, AQP3 ve tip I kollajen boyama ayrıca Hematoksilen-eozin boyama yapıldı. Bulgular: Genç ve yaşlı sıçanlarda immünohistokimyasal olarak AQP1, AQP3 ve tip I kollajen ekspresyonu gösterildi. Yaşlanma ile AQP1, AQP3 ve tip I kollajen miktarında azalma olduğu gözlendi. Tartışma: AQP1 ve AQP3 miktarındaki azalmanın kemik dokuda yaşa bağlı değişikliklerle ilgili olabileceği kanaatindeyiz.

Anahtar Kelimeler

Sıçan; Kemik Dokusu; Yaşlanma; Aquaporin 1; Aquaporin 3

Özet

Aim: Aquaporins are membrane channel proteins that transport water and ions. The amount of aquaporins in tissue changes with age. The amount of aquaporin-1 (AQP1) and aquaporin-3 (AQP3) is considered to decrease in cartilage tissue with age. However, no age-related change has been reported regarding the amount of aquaporins in bone tissue. In this study, our aim was to examine expressions of AQP1, AQP3, and type I collagen immunohistochemically in the bone tissues of young and old rats. Material and Method: Fourteen Wistar Albino rats were included in this study. Group I (n=7) consisted of young rats that were two months old, while Group II (n=7) consisted of old rats that were eighteen months old. Bone tissue (femur) was dissected and examined histopathologically and immunohistochemically. After routine histological procedure, sections at 4-5 μm thickness were obtained from tissues embedded in paraffin. Sections were stained immunohistochemically for AQP1, AQP3, and type I collagen as well as with hematoxylin-eosin. Results: Immunohistochemically, expressions of AQP1, AQP3, and type I collagen were demonstrated in both young and old rats. AQP1, AQP3, and type I collagen amounts were found to decrease with aging. Discussion: Our findings suggest that reduced amounts of AQP1 and AQP3 may be related to age-related changes in bone tissue.

Anahtar Kelimeler

Rat; Bone Tissue; Aging; Aquaporin 1; Aquaporin 3

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Introduction

Bone is composed of organic and inorganic material and water. Mineralized bone tissue contains a considerable amount of water. Twenty percent of the wet weight of spongious bone is composed of water [1]. The aforementioned rate declines to ten percent in mature cortical bone. Water in bone can be found in the mineral phase or organic phase (collagen and cement substance), or it can be in free form [2]. The majority of the water resides in various places such as microscopic pores of the lacuno-canalicular and Haversian systems, being found in different binding states [3]. Water provides a medium for the diffusion of ions and has a critical role in various physiological processes taking place in calcified tissues, like bone. Water also acts as a solvent for the transportation of small molecules within Haversian and lacuno-canalicular systems [4]. The maximum amount of water present in the vascular-lacuno-canalicular area constitutes about twelve percent of the total volume of bone [5]. Water is essential for physiological functions of the cell. Furthermore, entry of water into the cell occurs in three ways: diffusion, permeation, and transport channels. Water is transported along with other nutrients and metabolic products. The transportation of water through membranes is very important for the maintenance of normal cellular metabolism [6].

Water is not only confined to microscopic pores, whose number and size increase with aging, but is also present in extracellular bone matrix. The distribution of water within bone (either bound to collagen or minerals or free) changes over the lifetime. The amount of water in bone tissue decreases with the advance of skeletal growth and mineralization [5]. In addition, the increase in pore volume, which is a result of bone loss occurring with age, reduces the mechanical strength of the bone [7]. Understanding the distribution of water within bone tissue may help to explain the pathophysiology of fractures occurring in the elderly population [5].

Free form water fills the interconnected canalicular network of calcified matrix pores that are in connection with Haversian canals. This communication network is responsible for the two-way transportation of nutrients and waste products between vascular system and osteocytes. Furthermore, water channels also function as a carrier pathway for the flow of calcium and phosphate within the bone tissue [2].

Aquaporins (AQPs) are integral membrane proteins whose main function is to carry water through cellular membranes against the osmotic gradient. Since the first discovery of AQPs in red blood cells by Agre in 2004 [8], thirteen subtypes of AQPs (AQP 0-12) have been described in mammalian cells. All AQPs are similar with regard to membrane topological features." They are categorized in two subgroups based on amino acid resemblance and permeability to solutes: those facilitating water transport (AQP0, AQP1, AQP2, AQP4, AQP5, AQP8), and the aquaglyceroporins that facilitate the transport of water and neutral solutes, such as glycerol and urea (AQP3, AQP7, AQP9) [6,9,10].

AQP1 and AQP3 expressions have been found in human joint cartilage and intervertebral discs [11,12]. It has been suggested that the aforementioned aquaporins might play a role in metabolic water regulation in cartilaginous structures of weight-bearing joints, as well as in the pathophysiology of diseases such as osteoarthritis [11]. Also, it has been stated that AQP1 and AQP3 might have a role in age-related degeneration in intervertebral discs [12]. Although there are studies concerning the presence of AQP1 and AQP3 in tissues like joint cartilage and intervertebral disc, we did not encounter any study reporting on the presence and age-related changes of AQP1 and AQP3 in bone tissue. Considering the lack of previous studies on this topic, our aim was to determine the presence and age-related changes of AQP1 and AQP3 in bone tissue.

Material and Method

Animals

Fourteen Wistar Albino rats were included in this study. There were two groups of rats: one group consisted of young rats that were 2 months old (n=7), while the other group consisted of old rats that were 18 months old (n=7). All laboratory procedures applied on the rats conformed to the National Research Council Guide for the Care and Use of Laboratory Animals and to institutional guidelines. The study was approved by the local ethics committee (HADYEK-08). In order to evaluate their healthiness, all rats were followed for 3 days prior to the experiment, in a room with a 12 hour dark and 12 hour light cycle, at 20-23 degrees (OC) ambient temperature, where they could move about freely. They were fed with commercial rat feed and tap water.

Sample collection

Following anesthesia with ketamine/xylazine (50/10 mg/kg), the animals were sacrificed via exsanguination. For immunohistochemical and histopathological examinations, the bone tissue (femur) was removed and fixed in ten percent formalin solution for two days. Then, the tissues were decalcified in twenty percent EDTA solution for a two week period. After reaching adequate softness, bone tissues were washed with phosphate buffer solution. Following routine histological procedures, tissues were then embedded in paraffin.

Microscopic examination

Histological examination

Tissues were embedded in paraffin for the evaluation of morphological changes. Sections at 4-5 μm thickness were obtained from paraffin blocks and then stained with hematoxylin-eosin (H&E). Stained sections were examined under a Zeiss Axio Lab A1 light microscope.

Immunohistochemistry

Immunohistochemistry was performed according to the procedure previously described with minor modification [13]. 4-5 μm-thick serial sections were collected on poly-L-lysine-coated slides (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 56ºC. Tissue sections were de-paraffinized in xylene and rehydrated in a graded series of ethanol. Sections were then treated two times in a microwave oven in 10 mM citrate buffer, pH 6.0 for 2 minutes, and were left to cool for 15 minutes. After three washes in the phosphate buffered saline (PBS), endogenous peroxidase activity was quenched by 3% hydrogen peroxide in PBS for 20 minutes, and the sections were washed again three times in PBS. The sections were then incubated in a blocking serum (Ultra V Block, ScyTek Laboratories, Utah, USA)

for 10 minutes to block non-specific binding. Subsequently, sections were incubated overnight at 4°C with AQP3 rabbit polyclonal IgG (cat no: ab85903, 1: 200, Abcam, UK) and AQP1 rabbit polyclonal IgG (cat no: ab15080, Abcam, USA). The sections were then washed three times in PBS and incubated with biotinylated anti-rabbit (BA-1000; 1:400 Dilution; Vector Labora-tories) secondary antibodies for 45 minutes at room temperature. After three washes with PBS, the antigen–antibody complexes were detected using a streptavidin–peroxidase complex (TP-060-HL; LabVision, Fremont, CA, USA) for 15 minutes, followed by three rinses with PBS. Bound peroxidase was developed with 3-amino-9-ethylcarbazol (AEC) (ScyTek Laboratories, USA) chromogen, and sections were counterstained with Mayer's hematoxylin (ScyTek Laboratories, Utah, USA) and mounted with Permount (ScyTek Laboratories, Utah, USA) on glass slides. For controls, sections were treated with the appropriate isotype of rabbit and mouse IgGs. Photomicrographs were collected with a Zeiss microscope (Zeiss Axio Lab A1).

Evaluation of the immunohistochemistry

Evaluation of the immunohistochemical labeling was performed using H-SCORE analyses, as previously described [14]. The intensity of AQP1 and AQP3 immunoreactivity was semi-quantitatively evaluated using the following intensity categories: 0 (no staining), 1+ (weak but detectable staining), 2+ (moderate or distinct staining), and 3+ (intense staining). For each tissue, an H-SCORE value was derived by first calculating the sum of the percentages of cells that stained at each intensity category, and then multiplying that value by the weighted intensity of the staining using the formula H-SCORE=∑Pi(i+ l), where 'i' represents the intensity scores and 'Pi' is the corresponding percentage of the cells. On each slide, five randomly selected areas were evaluated under a light microscope (40 x objectives). The percentage of cells at each intensity within these areas was determined by two investigators, at different times, who were not informed about the type and source of the tissues. The average score of the observers was then used.

Type I collagen expression was evaluated immunohistochemically according to the method previously described by Kanter [15]. In this method, the density of immunoreactivity was evaluated at two sections for each animal, at 400x magnification, and using eight fields at each section. The evaluation was made according to the following classifications: absence (-), a few (+), medium (++), high (+++), and very high (++++).

Statistical analysis

Statistical calculations were performed using IBM-SPSS 20 for Windows. All data were presented in mean (±) standard deviations (SD). The comparisons between groups were analyzed with Mann-Whitney U test for AQP1, AQP3, and type 1 collagen immunohistochemistry. Statistical significance was defined as $p<0.05$.

Results

Results of histopathology

The bone tissue of rats was stained with hematoxylin and eosin (H&E) and examined using light microscopy (Figure 1). Bone tissue had a normal appearance in young rats. However, in old rats there were osteosclerotic changes on the femur's surface near the joint. There were patchy losses of trabecular bone in the metaphyseal areas of the femur, due to increased resorption. The distances between trabeculae increased as a result of the decrease in spongious bone tissue. External cortical thickness of the bone was markedly reduced. There were also resorption cavities within the cortical bone.

Results of immunohistochemistry

AQP1 and AQP3 protein expressions were demonstrated immunohistochemically in young and old rats (Figure 2 and Figure 3). In particular, bone marrow, endosteum, and vascular endothelium were markedly stained with AQP1. In addition, osteoblasts, osteoclasts, and osteocytes in the trabeculae were stained immunopositive for AQP1. With regard to staining for AQP3, osteoclasts and osteoblasts were stained more densely, and osteocytes were stained more weakly. H-scores for both AQP1 and AQP3 were significantly higher in young rats (Figure 4). In addition, according to immunohistochemical staining for type I collagen, young rats demonstrated a high level of (+++) type I collagen immunoreactivity, while old rats demonstrated decreased (++) immunoreactivity for type I collagen (p=0.029) (Figure 5).

Discussion

While minerals provide rigidity to the bone tissue, collagens give flexibility and the ability to absorb energy. The strength of the bone depends both on the amount of bone tissue and

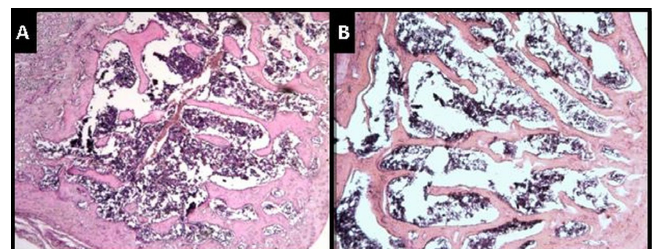

Figure 1. H&E staining in bone tissues of young and old rats x5. A-Bone tissue of young rats. B- Trabecular thinning and marked reduction in external cortical thickness of bone due to trabecular bone loss in old rats.

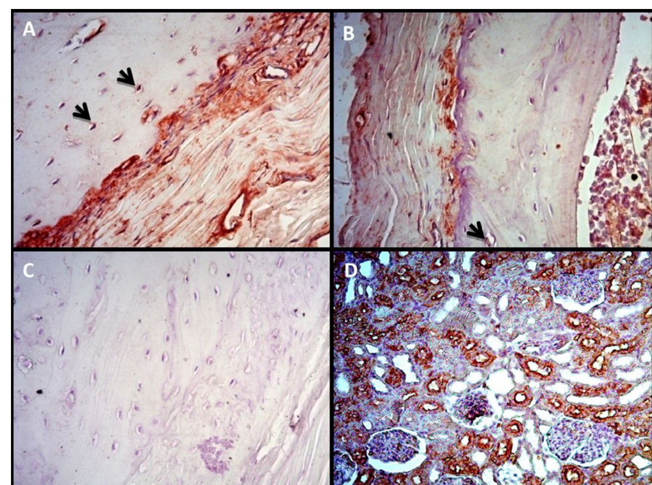

Figure 2. Immunohistochemical staining for AQP1 in bone tissues of rats x40. A- AQP1 immunoreactivity of young rats. Arrows point to immunopositive staining. B- AQP1 immunoreactivity of old rats. Arrow points to immunopositive staining. C- AQP1 immunoreactivity is not observed in negative control tissue. D- AQP1 immunoreactivity in positive control tissue (kidney).

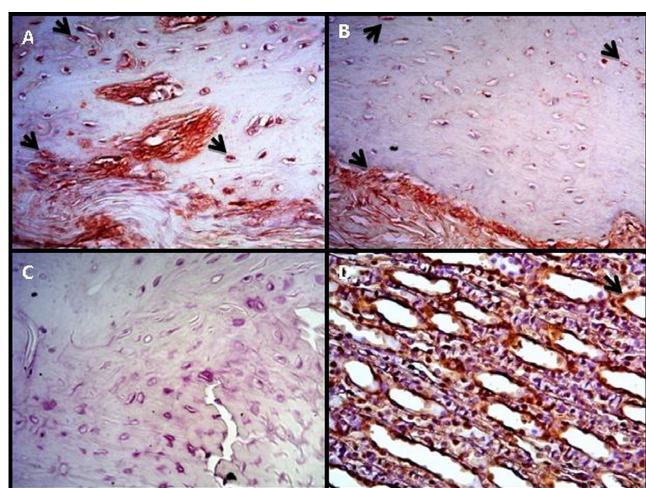

Figure 3. Immunohistochemical staining for AQP3 in bone tissues of rats x40. A- AQP3 immunoreactivity of young rats. Arrows point to immunopositive staining. B- AQP3 immunoreactivity of old rats. Arrows point to immunopositive staining. C- AQP3 immunoreactivity is not observed in negative control tissue. D- AQP3 immunoreactivity in positive control tissue (kidney).

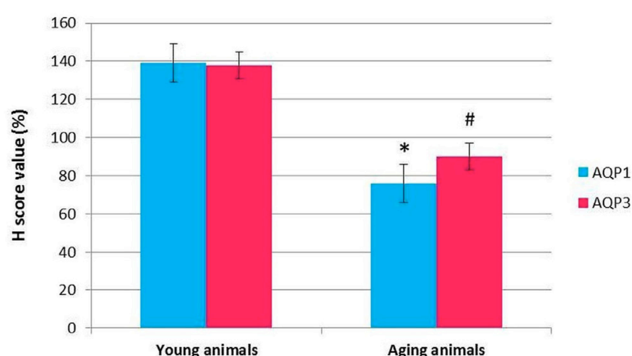

Figure 4. H-score values of AQP1 and AQP3 in young and old rats. The data are represented as mean±SD. *p< 0.0001, #p=0.001 old rats versus young rats.

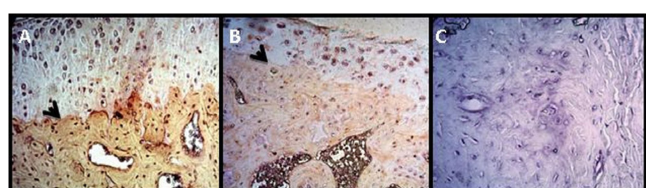

Figure 5. Immunohistochemical staining for type I collage in bone tissues of rats x40. A- Type I collagen immunoreactivity of young rats. Arrow points to immunopositive staining. B- Type I collagen immunoreactivity of old rats. Arrow points to immunopositive staining. C- Type I collagen immunoreactivity is not observed in negative control tissue.

the quality of the bone, which is characterized by its geometry, shape, microstructure, turnover, mineral, and collagen content. Collagens are a family of proteins present in connective tissue matrix. Furthermore, bone contains very large amounts of type I collagen, which makes up 95% of all collagen and 80% of all the proteins in bone tissue [16]. The amount of type I collagen decreases with age [7]. Age-related modifications that occur in collagen may disturb mechanical properties of the bone. Diseases that are related to collagen abnormalities and fragility in bone, such as osteogenesis imperfecta and osteoporosis, are indicative of the role of collagen cross-linking in bone [16]. In our study, the amount of type I collagen was also shown to decrease immunohistochemically with aging.

Aquaporins are responsible for the transport of water and certain small solutes. They are expressed to a great extent in many tissues and have a very important function in liquid homeostasis of the body [8]. Studies that have examined the structure of aquaporins show that they have a distinct, wide range of distribution with specific and important roles for each organ. AQP1 is expressed on barriers such as epithelium and endothelium, in choroid plexus, gallbladder, pancreatic ducts, intrahepatic cholangiocytes, hepatic ducts, placenta, amniotic membrane, cartilage, and synovium [17,18]. AQP1 is permeable to water and O_2, it prevents rapid volume deformations under osmotic stress, and it facilitates the diffusion of O_2 through plasma membranes. AQP1 has an important function in the regulation of cell volume and in the metabolic flow of water and matrix through membranes of chondrocytes. Immunohistochemical studies show that AQP1 is present on chondrocytes, synoviocytes at joints, and on vascular endothelial cells in synovial capillaries. The presence of AQP1 in chondrocytes is indicative of AQP1-mediated water transport through chondrocyte plasma membrane and synovial micro-vessels in weight-bearing joints [17].

In our study, AQP1 was present in the bone tissues of both young and old rats. AQP1 in particular was stained more intensely in the bone marrow, endosteum, and vascular endothelium; in addition, osteoblasts, osteoclasts, and osteocytes at the trabeculae were also stained immunopositive for AQP1.

In healthy joint tissue, AQP1 is expressed on synovial micro-vessels and synoviocytes [18]. In a study with fresh human cadavers, AQP1 expression was demonstrated immunohistochemically in the temporomandibular joint disc. Furthermore, it was stated that AQP1 had important functions in joint homeostasis [19]. It was reported that AQP1 might have important roles in the accumulation of vasogenic fluid and hydroarthrosis related to synovial inflammation via regulation of synovial homeostasis and chondrocyte volume [20]. It is considered that increased expression of AQP1 has a role in the pathogenesis of osteoarthritis (OA) [21]. In experimental OA models in rats, increased expression of AQP1 has been demonstrated both in vivo and in vitro in fibrochondrocytes in the meniscus tissue [22].

AQP3 is expressed in various tissues such as the kidney, respiratory epithelium, choroid plexus, prostate, pancreatic duct, endometrium, subchondral osteoblasts, and synovium [23]. In our study, immunohistochemical staining for AQP3 was more intense in osteoclasts and osteoblasts, and weaker in osteocytes. It has been considered that, in addition to its normal physiological functions, AQP3 might play a role in the pathophysiology of various diseases. Increased expression of AQP3 mRNA and the presence of AQP3 proteins in different localizations in OA cartilage indicate that they have important roles in OA pathogenesis [24].

Expression of aquaporin in various tissues changes with age. As a result of aging, changing expressions of AQP2 and AQP3 in the renal medulla [25], AQP1 and AQP3 in the intervertebral disc tissue [12,19], AQP1 in skin and in the choroid plexus of brain ventricles [26], AQP4 in the brain and the cochlea [27], AQP1 and AQP5 in the lungs [28], AQP1, AQP3, AQP4, AQP6, and AQP9 in the retina [29] were demonstrated. In our study AQP1 and AQP3 expression decreased with aging.

In our opinion, age-related alterations in aquaporin expression

and water-electrolyte metabolism have negative effects on bone structure. In addition, hyponatremia is known to be associated with osteoporosis and increased fracture risk. Increased arginine vasopressin (AVP) levels, which are frequently observed in the elderly, can cause water retention and hyponatremia, often stimulating calcium liberation from bones, leading to osteoporosis and increased fracture risk [14]. AQP9 expression has been shown to increase under normal physiological conditions during osteoclast biogenesis [9]; this has been reported to be important in the pathophysiology of various diseases that involve bone resorption [30]. However, there are no studies related to the association of AQP1 and AQP3 with pathologies of bone turnover. In our study, decreased expressions of AQP1 and AQP3 in bone tissue as a result of aging suggest that these two aquaporins play a role in the pathogenesis of osteoporosis that occurs with aging. When our current findings and previous studies have been evaluated, future studies should focus on the role of AQPs in bone tissue with aging.

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Competing interests

The authors declare that they have no competing interests.

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